Phenotypic responses to lifelong hypoxia in cichlids

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CHAPTER 6:

CLOSELY RELATED FISH SPECIES USE DIFFERENT STRATEGIES TO IMPROVE OXYGEN TRANSPORT AND METABOLISM UNDER CHRONIC HYPOXIA

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Abstract

We investigated the physiological responses of cichlids exposed to lifelong hypoxia. Broods of *Astatoreochromis alluaudi*, *Haplochromis ishmaeli* and a tilapia hybrid (*Oreochromis niloticus X O. mossambicus*) were split and exposed to normoxia (80-90% air saturation) and hypoxia (10% air saturation). Blood and white muscle tissue samples of adults were taken for analysis of physiological parameters for anaerobic metabolism (glycogen and total creatine levels and lactate dehydrogenase activity) and aerobic metabolism (blood haemoglobin concentration, haematocrit, intra-erythrocytic ATP and GTP levels and citrate synthase activity). Total creatine was not significantly different between normoxia-raised and hypoxia-raised siblings in all three species. Glycogen concentrations were significantly elevated in the white muscle of hypoxia-raised tilapia but not in that of *A. alluaudi* and *H. ishmaeli*. This indicates an increased anaerobic metabolic capacity in tilapia. The activity of the mitochondrial enzyme citrate synthase was not different between normoxia and hypoxia-raised tilapia but was significantly decreased in hypoxia-raised *A. alluaudi* and *H. ishmaeli*, indicating a decreased aerobic capacity. The haemoglobin and haematocrit levels were significantly increased in hypoxia-raised fish of all three species. In hypoxia-raised tilapia, intra-erythrocytic ATP and GTP levels were decreased suggesting an increase in blood oxygen affinity (a decrease in half-saturation oxygen tension, P_{50}) that safeguards sufficient O_2 loading of the haemoglobin under hypoxia. In contrast, no changes were found in *H. ishmaeli*, where different haemoglobin patterns were present in normoxia- and hypoxia-raised siblings. It is likely that these phenotypic differences in hypoxia-raised *H. ishmaeli* increase oxygen affinity of the whole blood. This study clearly shows that within closely related fish species, different strategies exist to cope with similar environmental changes.

Introduction

In general, hypoxia is considered to have detrimental effects on the survival of water breathers (Karlson *et al.*, 2002; Diaz and Rosenberg, 1995). The effects of hypoxia on fish are dependent on the duration of exposure. We distinguish short-term hypoxia from chronic hypoxia, short-term hypoxia lasting from hours up to several days, and chronic hypoxia lasts from a week to permanent hypoxia.

During short-term hypoxia, behavioural and regulatory changes result in a decrease in energy consumption, a maximised O_2 extraction, and an increase of anaerobic metabolism (Van den Thillart and Van Waarde, 1985). Fish that are exposed to short-term hypoxia, normally react with increased ventilation, a reduction in external activity and, if possible, aquatic surface respiration (Van den Thillart and Van Waarde, 1985; Chapman *et al.*, 2002). Upon immediate exposure to hypoxia, fish show stress responses that decrease tolerance. In contrast, when given time to habituate to the new environment, metabolic rates can be decreased (Randall, 1970) and stress responses avoided, resulting in higher hypoxia tolerance (Ultsch *et al.,* 1981). During more gradually induced short-term hypoxia, many fish species are able to reduce their energy consumption to below the standard metabolic rate, which is a determining factor for hypoxia tolerance. If the metabolic rate exceeds the maximum O_2 extraction, suppression of the standard metabolism follows, accompanied by activation of anaerobic metabolism to meet the total energy demand (Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). The ability to cope with short-term hypoxia is partly dependent on the coping strategy of the animal. Studies on sole, *Solea solea*, (Van den Thillart *et al.,* 1994) and rainbow trout, *Oncorhynchus mykiss*, (Van Raaij *et al*., 1996) show that fish can either react with tranquil behaviour, or escape responses. The latter is associated with, high levels of catecholamines and cortisol and low survival.

Exposure to chronic hypoxia can, through altered gene expression patterns, lead to the production of new enzymes, proteins etc. (Gracey *et al.*, 2001; Zhou *et al.*, 2001). Hypoxia exposure experiments with immature as well as adult fish, showed that survival of one to two months of hypoxia exposure is mainly based on the reduction of (aerobic) energy expenditure (Lomholt and Johansen, 1979; Johnston and Bernard, 1982a, b; Van den Thillart *et al.*, 1980; Zhou *et al.*, 2000; Wu *et al.*, 2003). After six weeks of chronic hypoxia exposure, the O_2 consumption of tench *Tinca tinca*, and carp was about 50% lower than that of normoxia-acclimated individuals (Lomholt and Johansen, 1979; Johnston & Bernard, 1982a). In the hypoxia-acclimated tench there was a 43-76% decrease in the perimeter of muscle capillaries and a

60% reduction in mitochondrial volume density (Johnston and Bernhard, 1982a).

In the hypoxia tolerant goldfish, *Carassius auratus*, chronic hypoxia exposure led to depression in protein synthesis in the liver, and elevated activity of enzymes that promote conservative use of glycogen stores in the muscles (Van den Thillart and Smit, 1984). In addition, stores of phospho-creatine (PCr) in the white muscles were significantly increased (Van den Thillart *et al.*, 1980). Chronic hypoxia exposure of young carp (35 g) resulted in decreases in serum testosterone, estradiol and triiodothyronine. These hormonal changes were associated with retarded gonadal development and reductions in spawning success, sperm motility, fertilisation success, hatching rate, and larval survival (Wu *et al.*, 2003). Indubitably, the metabolism of the fish in these studies was strongly limited by the ambient oxygen concentration. One might wonder whether this type of response enables lifelong survival. Theoretically, the best adaptation to chronic hypoxia permits uninhibited aerobic energy production and thus, a high oxygen extraction capacity under hypoxic conditions.

Most studies on chronic hypoxia concern exposure for several weeks to two months, performed on (semi)adult fish. Many fish species grow about a thousand-fold larger between the post-larval and adult stage. Conceivable phenotypic responses are larger if fish are exposed from very early stages onwards. Only few studies have addressed the effects of lifelong hypoxia exposure. Rutjes *et al.* (Chapter 2, 3) exposed cichlids to lifelong hypoxia (10% air saturation, 0.8 mg O_2 l^{-1}) starting at an age of about four weeks and a size of 1-1.5 cm standard length. They found routine oxygen consumption of hypoxia-raised (HR) cichlids at 10% air saturation (AS) to be the same or higher than that of normoxia raised (NR) siblings at 80% AS (7.2 mg O_2 l^{-1}). Also growth and routine activity levels were comparable between normoxia (NR) and hypoxia raised (HR) siblings. In contrast, at 10% AS, NR fish showed depression of metabolic rate and died after 12 hours.

Although HR fish at 10% AS showed normal non-depressed O_2 consumption rates it is likely that at peak activity levels, their aerobic metabolism will remain depressed by the low O_2 gradient. An increased anaerobic capacity would enable HR fish to maintain peak metabolic activity longer. In this way, behavioural activity such as fighting or mating is less compromised. Thus, increased levels of glycogen and PCr, which are the main substrates that fuel anaerobic metabolism, are required at chronic hypoxia.

As the P_{50} of the blood is close to the AS level of the inspired water, the gas exchange process has to operate within a small band width between O_2 loading and O_2 unloading. In *Oreochromis niloticus*, the P_{50} of whole blood (without CO_2)

is about 20 mm Hg at 25 ˚C (Verheyen *et al.*, 1985), which corresponds to about 13% AS. Thus, we can assume that the blood will only be partially oxygenated in the gills at 10% AS. (The oxygen carrying capacity stays the same when oxygen saturation decreases). Two common responses to hypoxia exposure in vertebrates are increases in blood Hb concentration (Frey *et al.*, 1998) and blood oxygen affinity (decreased P_{50}). In most fish, the Hb-O_2 affinity can be regulated by altering the intraerythrocytic concentration of organic phosphates, of which ATP and GTP are the most important (Weber, 1989, 1996; Val, 2000). Increases of organic phosphate concentrations reduce the blood-O_2 affinity. An alternative strategy to modulate oxygen affinity is to produce red blood cells with higher proportions of isoHb components with low P_{50}. Such alterations, albeit small, have been demonstrated in temperate fish following thermo- and hypoxia-acclimation (Houston and Cyr, 1974; Houston, 1980; Houston and Tun, 1986; Tun and Houston, 1986; Marinsky *et al.*, 1989). Cases of seasonal variation in the abundance of Hbs in fish erythrocytes are also known in several Amazon fish (Almeida-Val *et al.*, 1999).

Apart from altering fractions of already present Hbs, the P_{50} of the whole blood can also be reduced by production of novel Hbs. The production of new Hbs in fish is almost solely related to transitions in the O_2 availability, for instance during the shift from the water-breathing to the air-breathing stadium in air-breathing fish, or after birth in viviparous fish (Weber, 1990). Phenotypic adaptation by producing novel Hbs is not well explored. Studies adressing this phenomenon concern maximally several weeks of accimation and effects were not very large (Hattingh, 1976; Weber and Jensen, 1988, Weber, 1990).

To investigate the physiological responses of fish to lifelong hypoxia, we tested whether HR cichlids have increased stores of glycogen and PCr in the white muscles and increased enzyme activity that reflect a higher capacity to (an)aerobically produce energy. In addition, we tested whether increases in the Hb concentration, decreases in intraerythrocytic ATP and GTP occur, or changes in the presence of Hbs have occurred in the blood of cichlids that were exposed to lifelong hypoxia. Broods of three species of cichlids were split and raised under normoxia and hypoxia. After 15-19 months, blood and white muscle tissue were tested for specific adaptations to hypoxia.

Materials and methods

Raising and conditioning the animals

In this study we used broods of three species: a commercial strain of tilapia (*Oreochromis mossambicus* hybridised with *Oreochromis niloticus*), *Astatoreochromis alluaudi* and *Haplochromis (Labrochromis) ishmaeli*. The latter two are both haplochromine cichlids. The natural habitat of these species is different. Tilapias (both parental species of the hybrid) live in habitats with varying O_2 concentrations (Welcomme, 1967). The habitat of *A. alluaudi* includes both well-oxygenated streams as hypoxic wetlands (Greenwood, 1974; Witte, 1981). Oxygen concentrations in the habitat of *H. ishmaeli* are at a stable high level and hypoxic events are rare (Witte, 1981). Based on the differences in habitat, it is possible that the responses to chronic hypoxia differ between species.

The *A. alluaudi* and *H. ishmaeli* were obtained from breeding stocks in our laboratory. The breeding stocks are offspring of animals that were caught in the Mwanza Gulf in 1984 and have been bred in our laboratory since then. The tilapias were F1 offspring of animals obtained from the University of Nijmegen. In total, four broods were raised. One brood of each *A. alluaudi* and *H. ishmaeli* was raised in 1999 and a further brood of each *H. ishmaeli* and tilapia in 2002. Due to limitations in time and capacity, no second brood could be raised of *A. alluaudi* and tilapia. Nests of the *A. alluaudi* and *H. ishmaeli* were selected when animals were about 1.5 cm SL (about four weeks after fertilisation). Each nest was split randomly and raised under normoxia (NR) and hypoxia (HR). The tilapia specimens were 0.5-1 cm standard length when the brood was split (three weeks after fertilisation). All fish were raised for 15-19 months in 100-litre aquaria in the same climate room. There were no indications that sporadic deaths were related to hypoxia. Before adulthood, survival approximated 100% in both NR and HR groups. The water in the aquaria of the NR groups was kept at 80-90% air saturation (AS). The AS-level of the water of the HR groups was lowered stepwise to 10% AS in four weeks. The fish were kept at a temperature of 25.5 °C and a light-dark cycle of 12-12 hrs. The *A. alluaudi* and *H. ishmaeli* were given a diverse diet of flake food, frozen midge larvae, frozen zooplankton, and a mixture of pulverized shrimps, mussels and flake food. The tilapias were fed with cyclops, Duplarin (Dupla Aquaristik GmbH) and from about 5 cm SL onwards with commercial 4.5 mm tilapia pellets (Trouw Nutritions BV). The fish were raised in tanks of the same dimensions (45x50x50 cm). The glass tanks contained an extra compartment from where water was pumped into the animal compartment to ensure fast mixing with the inflow of hypoxic filtered water. Hypoxia was reached by a continuous inflow of

degassed water (6-9% AS) at a rate of 1-2 L/min. A stainless steel plate that was placed 3 cm below the water level prevented oxygen uptake from the air by the fish and by the circulating water. The oxygen level of the water was regulated by Applikon biocontrollers (ADI 1030) equipped with polarographic oxygen sensors (Applikon ZZ71202AP10), switching solenoid valves in line with air diffusers. Thus, air bubbling through the water in the extra compartment was initiated automatically when the oxygen level was below the setpoint, whereby the oxygen level in the animal chambers remained constant. Water in the normoxia and hypoxia tanks was continuously refreshed from the same biological filter system. Before flowing into the hypoxia tanks the water was degassed by a vacuum system as described by Van den Thillart and Smit (1984).

Sampling

To minimise possible handling effects that may increase blood catecholamine and cortisol levels and swelling of erythrocytes (Nikinmaa *et al.*, 1987), fish were caught from individual aquaria not more than once in a day. Per aquarium 2 fish were caught each Tuesday and Thursday between 10:00 and 11:00 hours and anaesthetised with MS222 (300 ppm). Animals were completely sedated within 1.5-2 minutes and blood was withdrawn within 3 minutes after sedation. Blood samples were taken from the caudal vein with 1-ml ice cold heparinised syringes (10,000 $IU \cdot ml^{-1}$) and kept on ice. Immediately after blood withdrawal, a sample of white muscle tissue was taken on both sides of the fish from the area between the 5^{th} and 10^{th} dorsal fin ray and above the lateral line. The tissue samples were instantly frozen with freeze clamps that were cooled in liquid nitrogen. The samples were stored in liquid nitrogen until further processing.

Measurements in the white muscle samples

The frozen muscle tissue samples were ground to a fine powder in a mortar that was cooled in liquid nitrogen. The powdered samples were stored in liquid nitrogen for further processing.

Portions of the sample were suspended in nine parts of a 0.1 M KH_2PO_4 buffer pH 7.4, vortexed and centrifuged for ten minutes at 21000 g. The supernatant was stored at -80°C and used for measurement of lactate dehydrogenase, pyruvate kinase and citrate synthase (CS) activities. The suspensions used for CS activity measurements were sonified for 10 seconds before vortexing. The LDH activity (EC 1.1.1.27) was determined spectrophotometrically by measuring the conversion of NADH into NAD^+ at 340 nm (Vassault, 1987). Citrate synthase (EC 4.1.3.7) was determined spectrophotometrically by measuring the conversion of $APAD^+$ into APADH at 340 nm (Stitt, 1984). To correct the enzyme activity for possible

differences in extraction efficiency, total protein content was measured, using a standard protein assay kit (BCA protein assay, #23225, Pierce).

Glycogen was measured from portions of the ground samples by complete hydrolysis into glucose with glucoamylase and spectrophotometrically determining the glucose concentration from the conversion of $NADP^+$ into NADPH at 340 nm (Keppler and Decker, 1988).

The concentration of creatine phosphate in the tissue samples was determined by HPLC, following the protocol of Harmsen *et al.* (1982). In addition the concentration of creatine was determined from the same samples according to Wahlefeld and Siedel (1985). To minimise the bias due to the sampling procedure in the present study, total creatine (creatine + creatine phosphate) was used as a measure.

Measurements in the blood samples

Separate aliquots of whole blood were used for the measurement of hematocrit (HCT), and concentrations of Hb, adenosine triphosphate (ATP) and guanosine triphosphate (GTP). For Hb measurements a standard reagent (Roche) for spectrophotometric Hb-determination was used. For nucleotide measurements, 50 µl of full blood was kept on ice for 10 minutes after admixture of 200 µl PCA solution (8% PCA, 10 mM EDTA, 4 mM NaF, 40% ethanol). The samples were sonified, remixed and resonified for 15 seconds and centrifuged for 10 minutes. The supernatant was separated and 200 µl was treated with 50 µl of a 3M K_3CO_2 solution and centrifuged again for 10 minutes. The supernatant was stored at -80°C, and later used for measurement of GTP and ATP by HPLC following the protocol of Harmsen *et al.* (1982).

The remaining blood (that was not used for measurement of Hb, HCT or ATP and GTP) was centrifuged five minutes at 10,000 g at 4ºC to separate cells and plasma. The erythrocyte pellet was resuspended in saline and centrifuged for 10 minutes after which the saline and red cells were separated. This was repeated twice after which the erythrocytes and plasma were stored at -80°C for further analysis.

Of the stored plasma samples, cortisol levels were measured using an enzyme immunoassay (Oxford Biomedical Research). The washed erythrocyte pellet was thawed and diluted twice with water. For qualitative determination of the isoHbs, isoelectric focussing of the Hb isomorphs was carried out using the PhastSysteml^{tm} (Pharmacia biotech) system on polyacrylamide gels in the 5-8 pH range. Marker proteins were applied as a reference. The separated Hbs were fixed and stained with Coomassie blue.

Statistics

Data were statistically analysed wit the program SPSS version 11.0. All data were normally distributed.

Results

Muscle Tissue

There were no significant differences in total creatine stores in *A. alluaudi* and *H. ishmaeli* between species nor between NR and HR siblings (one-sided Independent Samples T-tests, p-values >0.05).

In the white muscle samples, glycogen stores of HR tilapia were significantly greater than of NR siblings (one-sided Independent Samples T-test, p= 0.013). In *A. alluaudi* and *H. ishmaeli*, glycogen stores did not differ significantly between NR and HR siblings (one-sided Independent Samples T-test, p-values >0.05). The activity of LDH was not significantly different between NR and HR siblings of any of the three species (one-sided Independent Samples T-tests, p-values >0.05). When expressed as international units per gram tissue, CS activity differed significantly between NR and HR animals in *A. alluaudi* and *H. ishmaeli* but not in tilapia (one-sided Independent Samples T-tests, p-values respectively 0.002, 0.006 and 0.134).

Blood

The cortisol level was below 100 Ng L^{-1} in all animals and no significant differences were found between NR and HR animals (Independent Samples t-tests p-values >0.05; Table 1).

The Hb and Hct values were significantly higher in HR than in NR siblings of all three species (Table 1). In the *A. alluaudi* and *H. ishmaeli*, the differences in Hb and Hct were larger than in tilapia. No significant differences in MCHC were found between NR and HR siblings in any of the three species. ATP or GTP levels were not measured in the red cells of *A. alluaudi* and *H. ishmaeli* from 1999. In the *H. ishmaeli* from 2002, no differences in ATP and GTP levels were found between NR and HR siblings. (Independent Samples t-test, p=0.847 and 0.181). In tilapia, no significant differences in ATP were found. The GTP concentration however, was significantly lower in HR than in NR fish (Independent Samples t-test, p=0.706 and 0.001).

Thin layer isoelectric focussing was performed on 6-7 individuals of each NR and HR group per species. Of the nests of *H. ishmaeli* from both 1999 and 2002, runs were made of 6 individuals of each NR and HR group. Within each NR or HR group no clear individual variation in band patterns was found in any of the three species. In *A. alluaudi* and in tilapia, no differences were seen in the band patterns between the NR and HR group. All fish showed the same 16 isoHbs (Figure 1). In *H. ishmaeli* the NR animals showed 9 different Hbs while HR animals showed 10 different Hbs. Of the total of 14 mobilities that were found, the mobilities 2, 7, 9, 11 and 13 were absent in NR fish, while mobilities 5, 8, 11 and 14 were lacking in HR fish. Of the fractions that were present in both NR and HR *H. ishmaeli*, mobility 6 was

much more abundant in NR than in HR fish.

Discussion

Behaviour and stress

Both NR and HR fish were active and showed normal social interactions, indicating that the animals in were unstressed in both NR as well as HR groups. Differences in behaviour between both groups did exist. Mortality due to fighting of dominant males was higher in the NR than in HR groups. In addition, the HR fish were less gluttonous than NR siblings. The cortisol level of unstressed fish is generally below 100 $ng \cdot l^{-1}$ (Vianen et al, 2001, 2002). It has been shown that exposure of tilapia to stepwise decreasing AS levels, increased

Table 1: *Body weights and physiological correlates (± SD) for anaerobic and aerobic metabolism in blood and tissue samples from normoxia-raised (NR) and hypoxia-raised (HR) siblings of three species,* A. alluaudi, H. ishmaeli *and tilapia. Units and abbreviations: Hct = haematocrit (%), Hb = haemoglobin concentration (mM), MCHC = mean cellular haemoglobin concentration (Hb/Hct), ATP/Hb and GTP/HB = mM per mM Hb, cortisol (Ng* L^{-1}*), Glycogen = in equivalent of µM glucose* g^{-1} *tissue, Total creatine = µM* g^{-1} *tissue, LDH = lactate dehydrogenase (IU* g^{-1} *tissue), CS = citrate synthase (IU* g^{-1} *tissue). a = significant difference between NR and HR siblings at the 0.05 level b = significantly different from* A. alluaudi *at the 0.05 level c = significantly different from* H. ishmaeli *at the 0.05 level d = significantly different from tilapia at the 0.05 level.*

	NR *A. alluaudi*	HR *A. alluaudi*	NR *H. ishmaeli*	HR *H. ishmaeli*	NR tilapia	HR tilapia
Weight	21.4±2.1	17.8±1.5	19.9±5.7	19.7±4.9	118.3±50	94.0±42
Blood	**Blood**	**Blood**	**Blood**	**Blood**	**Blood**	**Blood**
Hct	31.3±3.5 **a**	44.47±5.1	31.2±4.6 **a**	44.80±8.62	34.4±5.6 **a**	39.4±4.1
Hb	5.0±1.5 **a**	6.8±1.6	4.9±1.0 **a**	5.8±1.8	5.9±0.6 **a**	6.8±0.5
MCHC	0.16±0.04	0.15±0.04	0.15±0.03	0.13±0.03	0.18±0.04	0.17±0.02
No. of Hbs	17	17	8	14	8	8
ATP/Hb	X	X	0.76±0.22	0.77±0.1	0.53±0.3	0.58±0.2
GTP/Hb	X	X	0.32±0.08 **d**	0.28±0.05	1.12±0.57 **a,c**	0.51±0.2
ATP/GTP	X	X	0.44±0.15 **d**	0.36±0.07	2.19±0.44 **a,c**	0.94±0.36
Cortisol	46.8±25.2 **d**	44.4±18.2	45.0±33.9 **d**	33.3±30.1	23.1±22.1 **b,c**	16.4±16.0
Tissue	**Tissue**	**Tissue**	**Tissue**	**Tissue**	**Tissue**	**Tissue**
Glycogen	15.6±5.9	14.0±2.9	16.9±6.9	15.13±8.8	17.7±5.8 **a**	28.3±11.9
Tot. Creatine	30.5±1.1	29.5±4.8	33.5±5.5	33.0±5.5	33.4±6.1	28.3±6.2
LDH	201.7±61.5	204.6±45.2	220.2±70.8	258.2±55.7	304.8±75.3	240.8±97.7
CS	2.64±0.05 **a**	2.00±0.78	2.85±1.01 **a**	2.12±0.44	1.61±0.43	1.46±0.25

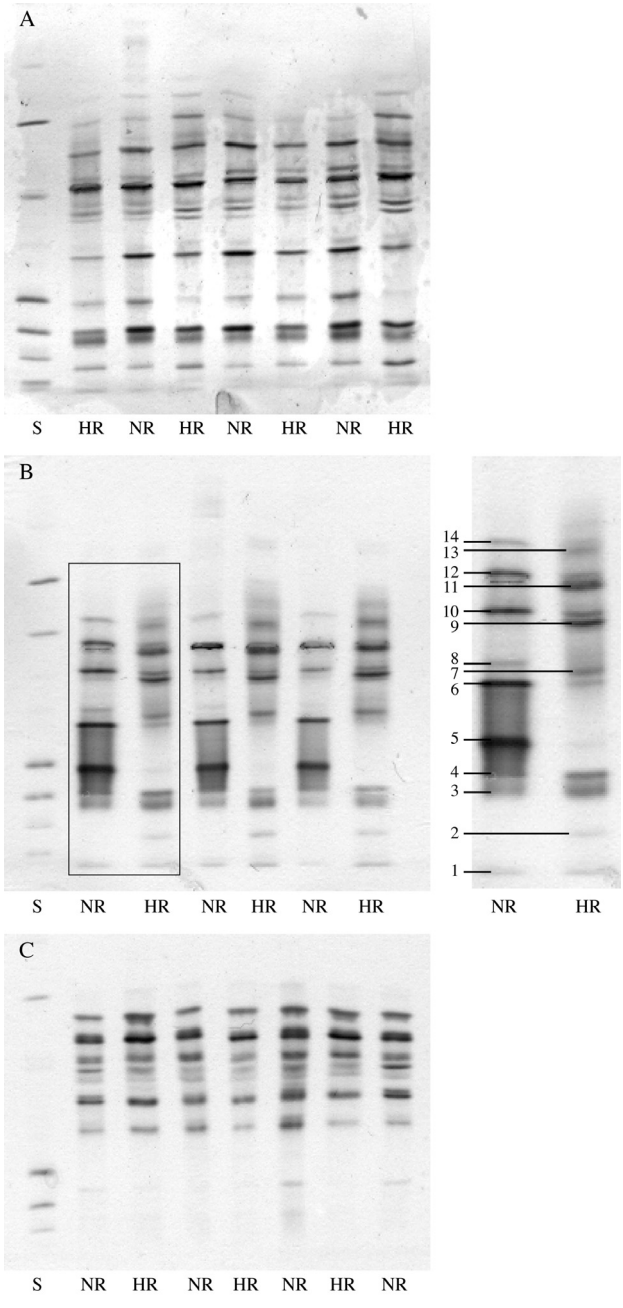

Figure 1: *Gel electrophoresis of blood samples of (A):* A. alluaudi*, (B):* H. ishmaeli *and (C): tilapia. S= standard protein mix, NR = normoxia-raised, HR = hypoxia-raised.* All A. alluaudi *and tilapia specimens showed the same band patterns, indicating that blood of NR and HR fish contained the same isohaemoglobins. Band patterns of NR and HR* H. ishmaeli *were different. In the NR H. ishmaeli bands 2, 7, 9, 11 and 13 were absent while in HR siblings bands 5, 8, 12 and 14 were absent.*

cortisol concentrations at and below 20% AS (Vianen *et al.*, 2002). Cortisol has a suppressing effect on the immune system and is associated with retarded growth (Wendelaar Bonga, 1997). Since growth was similar between NR and HR groups, elevated cortisol levels were not expected in the HR groups. Indeed, no significant differences in cortisol were found in our experiment between NR and HR animals (Table 1). We therefore conclude that10% AS was not a stressful situation for HR cichlids.

Metabolism

In general, glycogen stores in liver and white muscle appear to be higher in fish species that are relatively anoxia/hypoxia-tolerant such as tench, *Tinca tinca*, goldfish, *Carassius auratus*, and Crucian carp, *Carassius carassius*, than in relatively anoxia/hypoxia-intolerant species such as rainbow trout, *Oncorhynchus mykiss*, and cod, *Gadus morhua*, that have very low levels of muscle glycogen (Van den Thillart and Van Raaij, 1995). In *A. alluaudi*, *H. ishmaeli* and tilapia, the white muscle stores of glycogen were about 14-28 μM glucose per gram tissue, which is in the same range as covered by the above-mentioned species.

Glycogen concentrations in the white muscles were significantly elevated in HR tilapia but not in HR *A. alluaudi* and *H. ishmaeli* (Table 1). It supports the idea that HR tilapia compensate limitations in the O_2 consumption by increasing anaerobic capacity of the muscles during peak activity. However, in case of elevated anaerobic metabolism, an elevated activity of the enzyme LDH may also be expected, which was not the case. LDH converts pyruvate into lactate which is an important step in the anaerobic glycolysis. Greany *et al*. (1980) argued that white muscles already have a high glycolytic capacity and would therefore be pre-adapted to hypoxia, obviating the need for a capacity increase during chronic hypoxia.

The CS activity in the white muscles was similar between NR and HR tilapia, indicating an equally high aerobic capacity. However, in HR *A. alluaudi* and *H. ishmaeli*, CS activity was significantly reduced by 25%. Instead of compensations for limited aerobic metabolism at lifelong hypoxia, the unchanged anaerobic capacity and decreased CS activity indicate a reduction in overall maximum metabolic rate. At peak activity levels, for instance during territorial fights or when fleeing for predators, O_2 consumption of HR *A. alluaudi* and *H. ishmaeli* will be impaired and anaerobic energy stores depleted at lower metabolic rates than in NR siblings. We can conclude that acclimation of *A. alluaudi* and *H. ishmaeli* to lifelong hypoxia does not prevent impairment of maximum behavioural activity. This may have serious consequences for the ecology and survival of these species in the wild. However the decreased CS activity, did not affect routine activity

levels since O_2 consumption levels of HR cichlids was the same or higher than those of NR siblings (Chapter 3). CS is part of the TCA cycle and located within the mitochondria. It is known that in man, exercise at high altitude causes a reduction in the mitochondria content of the muscles (Hoppeler *et al*., 2003). Similarly, Johnston and Bernhard (1982a) showed that tench acclimated to 8.5% AS for six weeks have a decreased mitochondrial density in the white muscles. Thus, it is plausible that the decreased CS activity was a result of a reduction in the mean density of mitochondria in the white muscles.

Although, the creatine and glycogen stores in the white muscles did not differ between NR and HR siblings of *A. alluaudi* and *H. ishmaeli*, HR individuals of both species exhibited a marked increase in anoxia tolerance (Chapter 3). Possibly increased deposition of glycogen in the liver, an important storage organ of glycogen, could explain the increased anoxia tolerance in *A. alluaudi* and *H. ishmaeli*. Depletion of glycogen and PCr stores during hypoxia exposure in liver was demonstrated in goldfish Van den Thillart *et al.* (1980). Instead of attributing it to increased energy stores, the difference in anoxia tolerance between NR and HR fish could also be based on an increased ability regulate metabolic rate. For *A. alluaudi* this seems a likely possibility since particularly the HR individuals of this species, which were most hypoxia tolerant, reacted to anoxia exposure by lying on the bottom of the tank. Keeping this posture instead of an upright posture probably allows a greater depression of metabolic rate. Additionally, from visual observations it was very clear that those animals which showed the least external activity, were able to tolerate anoxia the longest (Chapter 3).

Responses of the haemoglobin system

Common responses in vertebrates to hypoxia are increases in Hb concentration occur as well as shifts in the P_{50} of the blood. However, hypoxia exposure did not change Hb and Hct levels in the hypoxia-tolerant carp and tench (Weber and Jensen, 1988). In HR fish from all three species used in this study, both total Hb and Hct levels increased but the mean cellular Hb concentration remained unchanged (Table 1), showing that the increase in Hb concentration was due to a higher amount of erythrocytes.

In vertebrates, organic phosphates are the most important allosteric effectors of Hb and provide a rapid means of adapting Hb function to tissue O_2 demand (Weber and Jensen, 1988; Weber, 1996; Val, 2000). In most fish, hypoxia exposure causes a decrease in organic phosphate concentration, mostly ATP and GTP, and is associated with an increase in Hb-O_2 affinity (Val, 1995). In most fish that have significant amounts of GTP in the red blood cells, the effect of GTP on Hb-O_2 affinity is stronger than that of ATP (Weber 1996). In tilapia Hb, the

effects of ATP and GTP are very similar (Babiker, 1985). As the P_{50} of the whole blood of tilapia is about 13% AS (20 mm Hg; Verheyen et al, 1995), a decrease in organic phosphate concentration would seem advantageous under hypoxia. In the red blood cells of NR tilapia used in this study, about twice as much GTP as ATP was found (Table 1). HR fish showed a 55% reduction in GTP levels resulting in equimolar ATP and GTP concentrations.

In contrast to tilapia, red blood cells of both NR as well as HR *H. ishmaeli* contained approximately equimolar concentrations of ATP and GTP. Apparently *H. ishmaeli* does not use organic phosphates to increase its Hb-O_2 affinity, which contrasts with the hypoxic response generally seen in fish. However, routine O_2 consumption rates in this species were similar between NR and HR siblings (Chapter 3). Apparently, a decrease in the P_{50} is regulated differently in this species.

Isoelectric focussing of the Hbs of *A. alluaudi* and tilapia showed no differences in Hb multiplicity between NR and HR siblings. However, in both batches of *H. ishmaeli* that were used, clear differences were observed between NR and HR siblings (Figure 1). HR fish lacked four isoHbs that were present in NR siblings. However, five new Hbs were seen that were lacking in NR *H. ishmaeli*. This pattern was consistent for all NR and HR fish examined. This clear cut difference has to our knowledge not earlier been observed in fish. The presence of such a distinct Hb pattern in hypoxia-raised *H. ishmaeli*, indicates that a regulatory mechanism is involved. These changes may well have resulted in a decreased P_{50} of the whole blood, thereby increasing the Hb-O_2 loading in the gills at 10% AS.

Production of different Hbs in different environments was up till now only known from animals that undergo drastic changes in ontogenetic development, for instance birth in humans, viviparity in fish, or water to air transitions in amphibians (Weber, 1990; 1994) Thus, the ability of *H. ishmaeli* to produce different Hbs under different environmental conditions is quite unique in the respect that it seems to be a real phenotypically plastic trait that is not bound to ontogenetic shifts.

The hypoxia response of tilapia *viz.* decreasing the concentration of GTP that increases Hb-O_2 affinity fits very well for life in environments where O_2 concentrations fluctuate (hours-weeks). The concentration of organic phosphates in the red blood cells can be altered in a matter of minutes to a few hours (Val, 2000). This fits well with the wide dispersion and variety of O_2 concentrations that is found in the habitats *O. niloticus* and *O. mossambicus* (Welcomme, 1967, Trewavas, 1983). The hypoxia responses of *H. ishmaeli viz.* a switch between phenotypes with different Hb composition of the blood, can only be used in environments with

stable O_2 concentrations (months-years) since a large fraction of the erythrocyte pool needs to be replaced for the response to be effective. Considering the life span of red blood cells, this can take several months. The habitat in which *H. ishmaeli* was found before the introduction of lake-wide chronic hypoxia, was thought to be a stable normoxic environment (Van Oijen *et al*., 1981). Apparently, in the evolutionary past of *H. ishmaeli*, selective forces (occurrence of hypoxia) must have occurred, favouring animals with the capacity to develop distinct alternative phenotypes at different stable AS levels. Possibly this capacity evolved during the desiccation of Lake Victoria 14,000 years ago (^{14}C 12,400 years; Johnson et al., 1996). In the present, *H. ishmaeli* could benefit from the increased occurrence of hypoxia in Lake Victoria and settle in hypoxic areas that have become less suitable habitats for other species. Although each of the three species of cichlids here studied cope with hypoxia very well, this study clearly shows that very closely related fish species deploy widely different strategies to cope with the same environmental challenges.